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## Interaction and Replication Activation of Genotype I and II Hepatitis Delta Antigens

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The nucleotide sequences of hepatitis D viruses (HDV) vary 5 to 14% among isolates of the same genotype and 23 to 34% among different genotypes. The only viral-genome-encoded antigen, hepatitis delta antigen (HDAg), has two forms that differ in size. The small HDAg (HDAg-S) trans-activates viral replication, while the large form (HDAg-L) is essential for viral assembly. Previously, it has been shown that the packaging efficiency of HDAg-L is higher for genotype I than for genotype II. In this study, the question of whether other functional properties of the HDAgs are affected by genotype differences is addressed. By coexpression of the two antigens in HuH-7 cells followed by specific antibody precipitation, it was found that HDAgs of different origins interacted without genotypic discrimination. Moreover, in the presence of hepatitis B virus surface antigen, HDAg-S was incorporated into virion-like particles through interaction with HDAg-L without genotype restriction. As to the differences in replication activation of genotype I HDV RNA, all HDAg-S clones tested had some transactivation activity, and this activity varied greatly among isolates. As to the support of HDV genotype II replication, only clones of HDAg-S from genotype II showed trans-activation activity, and this activity also varied among isolates. In conclusion, genotype has no effect on HDAg interaction and genotype per se only partly predicts how much the HDAg-S of an HDV isolate affects the replication of a second HDV isolate.

The hepatitis D virus (HDV) is a small satellite virus of hepatitis B virus (HBV) (17, 30) and can be experimentally transmitted to chimpanzees and woodchucks (24, 25). Coinfection or superinfection with HBV and HDV may cause severe liver disease in humans (10, 11, 26, 33, 36, 40). However, some chronically HDV-infected subjects with viremia may appear asymptomatic and have normal to mildly elevated serum transaminase levels (41).

The HDV viral particle is composed of HBV surface antigen (HBsAg), a single-stranded circular 1.7-kb HDV RNA, and two forms of the hepatitis delta antigen (HDAg). The large form of HDAg (HDAg-L) is identical to the small one except for a 19-amino-acid extension at the C terminus. There is a CXXX motif in this C-terminal extension, which results in HDAg-L being isoprenylated, but not HDAg-S. Because of these molecular characteristics, HDAg-S and HDAg-L share some properties but differ in others (22). Both HDAg-S and HDAg-L form oligomers (32) and interact with viral RNA (7, 20). However, only HDAg-L plays a critical role in viral assembly (5, 8, 22). HDAg-L also suppresses viral replication (8, 21), while HDAg-S can *trans*-activate HDV RNA replication (16, 29, 43).

Different isolates of HDV vary in their nucleotide sequences. Most studies of HDV replication have focused on genotype I due to the availability of cDNA clones. Viruses of this genotype have been widely found in North America, Europe, Africa, east and west Asia, and the South Pacific (28, 44). However, HDV isolates phylogenetically different from genotype I from other geographical locations have been identified,

and they are classified into genotypes II and III (2, 15, 37, 38). The viral nucleotide sequences vary by 23 to 34% among isolates of different genotypes and about 5 to 14% among those of the same genotype (28, 37). It has been suggested that variations in nucleotide sequences are responsible for the pathogenic differences between HDV infections caused by different genotypes. The genotype III HDV has been associated with severe forms of hepatitis that frequently occur in northern South America (2), whereas the genotype II HDV, which has been isolated in Japan and Taiwan (15, 38), has been associated with less-aggressive disease than genotype I (38).

Both HDAg-S and HDAg-L contain a stretch of amino acids named the coiled-coil domain (27, 32). In studies using two-dimensional <sup>1</sup>H nuclear magnetic resonance (9) and circular dichroism techniques (9, 27), synthetic peptides covering this region have been shown to form an alpha-helix structure in solution. The molecules can associate into a dimeric structure detectable by gel filtration chromatography (27). Moreover, the high-resolution crystal structure of a synthetic HDAg peptide (residues 12 to 60) has been determined (45). The results show that HDAgs may dimerize through an antiparallel coiled-coil form. These dimers then associate further to form octamers through the residues in the coiled-coil domain and the residues C-terminal to this region.

This coiled-coil interaction, at least in part, may contribute to the observed functions of HDAgs (22). Oligomerization of HDAg-S is required for the *trans*-activation of viral RNA replication (43, 45). However, HDAg-S by itself is insufficient for incorporation into the viral particle (5, 8). It needs the help of HDAg-L in the copackaging process, and this interaction is mediated by the coiled-coil domains between the molecules. The same interaction also results in the *trans*-dominant suppression of HDAg-L on HDV replication (5, 43). Previously, functional incompatibility between the different genotypic

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HDAgs has been observed. HDAg-S of genotype III is unable to support viral RNA replication of HDV genotype I, and this is true for the reciprocal pairing as well (3). These observations raised the interesting issue of whether HDAgs of different genotypes vary in their biochemical properties.

Recently, we have shown that the package efficiency of genotype I HDV is generally higher than that of genotype II and that the C-terminal 19-residue region of HDAg-L plays a key role in this aspect (13). Here, we addressed whether oligomerization of HDAg is genotypically restricted and whether different HDAg-S molecules have distinct *trans*-activation properties in HDV replication.

### MATERIALS AND METHODS

Plasmids for HDAg expression. The cDNA fragments encoding HDAg were obtained by reverse transcription-PCR from patients infected with HDV (38). The obtained cDNA fragments were ligated into commercial TA cloning vector pCRII (Invitrogen). The inserted segments in the plasmids were completely sequenced and cloned into XbaI/SphI- or XbaI/PstI-digested pCMV-EBNA (Clontech). TW1629, TW2577, TW2683, and TW1435 are genotype I isolates, and TW857, TW2476, TW3937, and TW2479 are isolates of the subgroup IIa of genotype II (37). To be concise, HDAg-S and HDAg-L derived from the TW2577 isolate are referred to as 25S and 25L, respectively. Similarly, 16S and 16L, 83S and 83L, 14S and 14L, 8S and 8L, 24S and 24L, 39S and 39L, and 79S and 79L represent the corresponding HDAgs derived from isolates TW1629, TW2683, TW1435, TW857, TW2476, TW3937, and TW2479, respectively, obtained from patients' sera. The plasmid pCMV-S, which contains the HDAg-S open reading frame (ORF), coding for Kuo-S, was derived from pSVL-S (16). The chimeric constructs derived from genotype I 16S and 25S were obtained by switching cDNA fragments between 16S and 25S by using the EcoRI and SmaI sites that are shared by the two isolates. The chimeric constructs derived from 24S and 39S of genotype II were obtained similarly by using the shared StuI and NaeI sites of 24S and 39S. Clones were screened by restriction digestion and confirmed by DNA sequencing.

Plasmids for HDV genome replication and other applications. Plasmid pCD-m2G contains a tandem dimer of the mutated HDV cDNA (1.7-kb XbaI fragment, genomic sense) with a 2-base deletion in the HDAg ORF (31). Under the control of the cytomegalovirus promoter, pCD-m2G transcribes nonreplicating genotype I HDV genomic RNA. As no HDAg-S is produced, the plasmid requires a functionally active HDAg-S in trans for viral replication.

To prepare similar clones containing the genotype II HDV genome, a serum sample from TW2479 was used for viral RNA isolation. After subsequent reverse transcription-PCR to generate HDV cDNAs, the overlapping subgenomic PCR products were assembled in pCRII (Invitrogen), with a reference to the *Xba*I site at nucleotide 783. Then, by a strategy of two-copy insertion, the 1.7-kb *Xba*I fragment was isolated and inserted as a tandem repeat into the *Xba*I site of pcDNA3.1(-) (Invitrogen), resulting in pCD-79D2G. To create a construct defective in HDAg-S synthesis but maintaining most of the RNA secondary structure (42), an adenosine was inserted between positions 1583 and 1584 of the HDV genome by site-directed mutagenesis. Thereby, a stop codon was introduced at codon 5 of the HDAg-S ORF, and the frame thereafter was shifted. The PCR-amplified fragment containing this mutation was then inserted in a tandem repeat into the *Xba*I site of pcDNA3.1(-) to generate pCD-79mD2G.

Plasmids pG3-Dg and pG3-79Dg were used for in vitro transcription with T7 RNA polymerase to generate RNA probes for detection of antigenomic HDV RNA; they were obtained by ligating the 1.7-kb *Xba*I fragments of HDV cDNA, separately derived from pSVL-D3 (16) and pCD-79D2G, into *Xba*I sites of pGEM-3Zf(-) (Promega).

Again, all the above clones were screened by restriction digestion and confirmed by DNA sequencing. Plasmid pS1X, encoding the three forms of HBsAg (4), was used in the cotransfection with HDAg-L expression plasmids to produce virion-like particles (VLPs) as previously described (13).

**Specific antibodies.** Antibodies against T1C (KPWDILFPADPPFSPQSCR PQ) and T2C (KPWVDPSPPQQRLPLLECTPQ) have been described previously (14). Monoclonal antibody (MAb) HP6A1 has also been reported previously (12). This MAb reacts strain specifically with residues 4 to 10 of the HDAg of the TW2577 isolate and does not cross-react with the HDAg of other isolates of genotypes I and II.

Cell transfection, immunoprecipitation, and Western blotting. HuH-7 cells (23) were used in all DNA transfections. Maintenance of cells and transfection of DNA by the calcium phosphate-DNA coprecipitation method were carried out as previously described (14, 34, 35). In general, cells were seeded onto a 60-mm-diameter dish at 70% confluence 1 day prior to transfection. After transfection with 10 µg of DNA, the cells were incubated for an additional 20 h. When cotransfected, plasmids were kept at equal amounts except where noted otherwise. The medium was then replaced at 3-day intervals thereafter. For transient expression of HDAg, the cells were harvested at day 2 posttransfection. Harvested cells were lysed in NET buffer, containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). Immunoprecipitation and analysis of the immunocomplexes by Western blotting were carried out as previously described (14, 34, 35). Pooled sera from HDV-infected individuals that cross-reacted well with HDAgs of both genotypes were used to detect all HDV antigens. The secondary antibody used was a horseradish peroxidase (HRP)-conjugated goat anti-human antibody (Sigma). When primary antibodies were derived from a rabbit, the immunochemical reactions were similarly carried out except that the secondary antibody used was goat anti-rabbit immunoglobulin G (IgG; Sigma). The HBsAgs were detected with MAb A10F1 (19), which was in turn reacted with HRP-conjugated goat anti-mouse IgG. Membranes were all finally developed with a Western blot chemiluminescence reagent (NEN Life Science).

RNA analysis. Total cellular RNA from transfected HuH-7 cells was isolated by TRIzol reagent (Invitrogen). Purification procedures were performed according to the manufacturer's instructions. RNA (15  $\mu g$ ) was then separated on 1.2% agarose gels containing 2.2 M formaldehyde and transferred to Immobilon-Ny+membranes (Millipore). After fixation by UV illumination, the membrane was hybridized with  $[\alpha^{-32}P]CTP-labeled HDV strand-specific riboprobes transcribed from HindIII-digested pG3-Dg or PstI-digested pG3-79Dg. Hybridization was performed at 68°C with HYB-9 hybridization solution (Gentra). The membranes were washed with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% SDS at 75°C and then exposed to X-ray films. The amount of probes bound on membranes was indirectly measured by phosphorimagery using ImageQuant (version 5.2; Molecular Dynamics). To control for an equal loading of samples, hybridization with a <math display="inline">[\alpha^{-32}P]dCTP$ -labeled glyceraldehyde-3-phosphate dehydrogenase probe was performed similarly except for the temperature of hybridization and washing, which was set at 60°C.

**Nucleotide sequence accession number.** The genomic sequence named TWD2479-12, containing 1,680 nucleotides, was deposited in GenBank with an accession number of AY261457.

### **RESULTS**

Complex formation between HDAg-L and HDAg-S from different genotypes. The amino acid sequences of five genotype I HDAgs (Kuo [16], 16, 25, 83, and 14) and four genotype IIa HDAgs (8, 24, 39, and 79) were aligned and are shown in Fig. 1. These sequences vary by about 10% among isolates of the same genotype; this variation increases to about 40% if sequences of different genotypes are compared. The genotypic differences among the HDAgs scatter along the entire sequence, but two major clusters can be identified. One is located in the N-terminal region (residues 3 to 40) and shows a 45 to 50% amino acid difference. This overlaps with the coiled-coil domain critical for HDAg oligomerization (17). The second is located at the C terminus (residues 196 to 214), and is present in HDAg-L but absent in HDAg-S; this region shows an amino acid sequence divergence of up to 74%.

To distinguish closely related HDV antigens, genotype-specific antibodies (anti-T1C and anti-T2C) directed to the C-terminal 19 residues of HDAg-L (14) were used. In a Western blot, anti-T1C reacts specifically with HDAg-L of genotype I but not with that of genotype II. Anti-T1C does not react with HDAg-S from any isolate because of the lack of the appropriate antigenic peptide (14). Coexpression of different HDAg molecules in HuH-7 cells was carried out by cotransfection with plasmids coding for the antigens. The HDAgs expressed

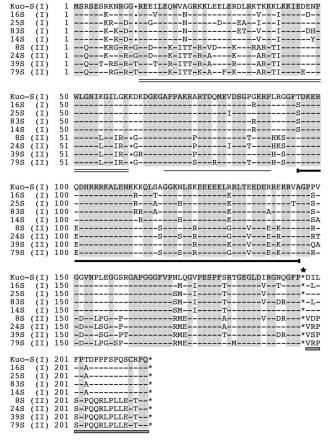


FIG. 1. Comparison of HDAg sequences of genotypes I and II. Shaded areas, identical amino acids among all isolates; dashes, residues identical to those of Kuo-S (16); dots, gaps introduced to produce an optimal alignment. The functional domains (17, 22) highlighted are the oligomerization domain (double lines), the nucleus localization sequence (single line), the RNA binding domain (thick line), and the package signal in HDAg-L (hatched box). \*, position corresponding to the stop codon for HDAg-S.

in the HuH-7 cell lysates were then bound to antibodies previously captured by protein A-agarose. The precipitates were dissolved in SDS sample buffer and analyzed for the presence of the HDAgs by Western blotting using pooled anti-HDVpositive human sera (Fig. 2A). Anti-T1C precipitated the antigens from 25L (data not shown; see Fig. 3, lane 1) but not from 25S, 24L, and 24S (Fig. 2A, lanes 2 and 4). When coexpressed with 25L (lane 1), 25S was detected in the precipitates. This finding indicated that 25S complexes with 25L, and this resulted in their coprecipitation by the protein A-bound anti-T1C. Since an interaction between the large and small forms of HDAg produced by genotype I HDV has been previously established (5), these results serve well as a positive control. Following this, coexpression of 24S and 25L in HuH-7 cells was used to test the cross-genotypic interaction of HDAgs. The results from Fig. 2A, lane 3, indicated that, although 24S did not react with anti-T1C, it was detected in the anti-T1C-25L immunocomplexes. Therefore, 24S must form complexes with 25L. Figure 2B demonstrates the reciprocal interaction of 25S with 24L. In similar experiments using different isolates of the same genotype II (i.e., 24S and 24L replaced with 8S and 8L,

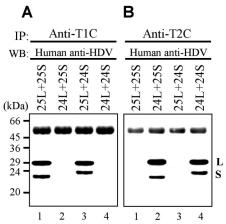


FIG. 2. Interactions of HDAg-L and HDAg-S between genotypes I and II. Interaction was demonstrated in transfected HuH-7 cells expressing different HDAgs. HDAgs in cell lysates were precipitated (IP) by genotype-specific antibodies bound to protein A-agarose. Proteins in the precipitates were then analyzed for HDAg by Western blotting (WB) using human anti-HDV without genotypic specificity. The genotype-specific antibodies (12) were anti-T1C, specific to the HDAg-L of genotype I (A), and anti-T2C, specific to the HDAg-L of genotype II (B). 25L and 25S, genotype I; 24L and 24S, genotype II; L, HDAg-L; S, HDAg-S.

respectively) identical observations were made (data not shown). Taken together, this showed that HDAg-S interacted with HDAg-L regardless of genotypic differences.

Complex formation between genotypically different HDAg-L molecules. The C-terminal region of HDAg-L differs by up to 74% between genotypes I and II. Whether this striking difference affects the mutual interaction between HDAg-Ls was examined next. Anti-T1C was used to precipitate 25L, and the precipitants were analyzed by Western blotting using human anti-HDV to detect all HDAgs (Fig. 3A, lanes 1 and 2) and anti-T2C to detect 8L only (Fig. 3B, lanes 1 and 2). When 25L was expressed alone, it was precipitated by anti-T1C, as shown

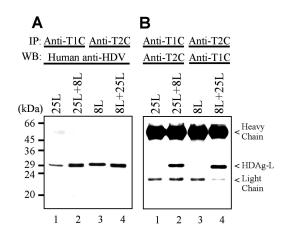


FIG. 3. Interactions between the HDAg-L molecules of different genotypes. Coexpression of the HDAg-L molecules in HuH-7 cells and immunoprecipitation (IP) of antigens in the cell lysates were carried out as described in the legend to Fig. 2. Verification of HDAg-L in precipitates was carried out by Western blotting (WB) using human anti-HDV- (A) and anti-HDAg-L (B)-specific antibodies. See the legend to Fig. 2 for antibody specificities. 25L, genotype I; 8L, genotype II.

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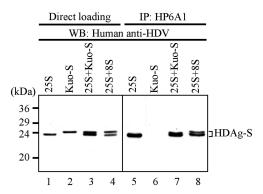


FIG. 4. Interactions between the HDAg-S molecules of genotypes I and II. Analyses of HDAg interactions were carried out as described in the legend to Fig. 2 except that MAb HP6A1 was used in the immunoprecipitation. MAb HP6A1 has a specificity such that it reacts only with 25L and 25S (12). Note that 25S migrated slightly faster than Kuo-S and 8S in SDS-polyacrylamide gel electrophoresis gel, presumably due to sequence variation.

by its positive detection with human anti-HDV (Fig. 3A, lane 1) and negative detection with anti-T2C (Fig. 3B, lane 1). When 25L and 8L were coexpressed, the antigens in the precipitant were detected in blots not only by human anti-HDV (Fig. 3A, lane 2) but also by anti-T2C (Fig. 3B, lane 2). Since anti-T2C is specific to genotype II HDAg-L and does not react with 25L, the immunodetected antigen in lane 2 of Fig. 3B must represent 8L. In a reciprocal experiment (Fig. 3B, lanes 3 and 4), anti-T2C did not precipitate 25L, and the 25L molecule detected by anti-T1C in lane 4 must be indirectly pulled down by interacting with 8L. Therefore, HDAg-L molecules associated with each other regardless of genotype differences.

Complex formation between genotypically different molecules of HDAg-S. As observed in Fig. 1, no continuous stretches of amino acids in the HDAg-Ss show any specific marked difference between genotypes I and II. Such antigens, with scattered variation, are difficult to distinguish by polyclonal antibodies. To circumvent this difficulty, the available MAb HP6A1, which binds strain specifically to 25S and 25L (residues 4 to 10) but not to other HDAg molecules, was used (12). Typical results are shown in Fig. 4. In lanes 1 to 4, samples from the plasmid-transfected HuH-7 lysates were directly loaded onto SDS-polyacrylamide gel electrophoresis gel and analyzed for all expressed HDAgs with human anti-HDV. The results showed that 25S moved slightly faster than other HDAg-Ss. Therefore, these HDV antigens were resolvable when coexpressed in the same preparation (lanes 3 and 4). After precipitation with HP6A1, 25S (lane 5), but not Kuo-S (lane 6), was detected in the immunoprecipitates by human anti-HDV. In the coexpression experiment, Kuo-S and 8S were readily coprecipitated with 25S and detected in the immunoprecipitates (lanes 7 and 8). These findings supported the previous results that complexes are formed between the different HDAg-Ss. Therefore, neither strain restriction nor genotype incompatibility was observed for interactions between different molecules of HDAg-S.

Copackage of HDAg-S with HDAg-L without genotypic specificity. An alternative approach that allows demonstration of the association of two HDAg antigens is to examine the VLPs released into the culture media when HBsAg is coexpressed

(8). In these experiments, a clone of HDAg-L (i.e., 16L) was paired with each HDAg-S from the eight clones as shown in Fig. 5. The harvested particles from the culture media were analyzed by Western blotting using human anti-HDV. Without coexpression with HDAg-L, HDAg-S alone was not incorporated into the HBsAg particles (Fig. 5C, lane 1), a result consistent with a previous report (8). The success of the cotransfection of three expression plasmids is shown by the finding of HDAgs (Fig. 5A) and HBsAg in the harvested particles (Fig. 5B). More importantly, HDAg-S from every isolate was detected in the harvested particles (Fig. 5C, lanes 2 to 9). These results strengthened the above hypothesis that HDAg-S and HDAg-L interacted with each other without genotypic specificity.

# trans-activation of HDAg-Ss on HDV replication of genotype I. The most varied region among HDAg-S molecules of genotypes I and II is located in the coiled-coil region (Fig. 1). Previously, functional incompatibility of genotype I and III HDAg-Ss has been observed (3). In Taiwan, both genotype I and II HDVs have been found (38), and mixed-genotype infections with HDV were known to exist in some subjects (39). It is thus of medical relevance to examine whether there is functional compatibility between HDAg-Ss of genotypes I and

To address this issue, a construct (pCD-m2G) that is competent to express genotype I HDV RNA intracellularly but defective for HDAg synthesis was used (31). Transfection of HuH-7 cells with pCD-m2G and a vector control (pcDNA3.1) yielded no sign of HDV RNA replication, as determined by the

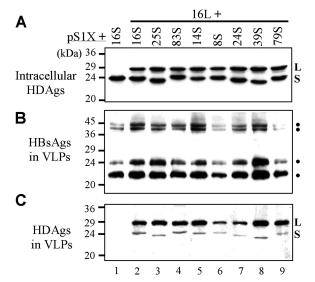


FIG. 5. Different genotypes of HDAg-S are copackaged with HDAg-L in VLPs. HuH-7 cells in 10-cm-diameter dishes were cotransfected with 7  $\mu g$  (each dish) of plasmids encoding HDAg-L (16L), a given HDAg-S, and HBsAg (from pS1X). Both cells and spent media were collected on day 3 posttransfection. HBsAg-packaged particles were concentrated from the spent media by centrifugation through a 20% sucrose cushion. (A) The cell lysates were directly analyzed for intracellular HDAg expression by Western blotting using human anti-HDV. (B and C) The pelleted particles were dissolved in the SDS sample buffer and analyzed for extracellular HBsAgs (B) and HDAg (C) by Western blotting. L, HDAg-L; S, HDAg-S; dots, different forms of HBsAg.

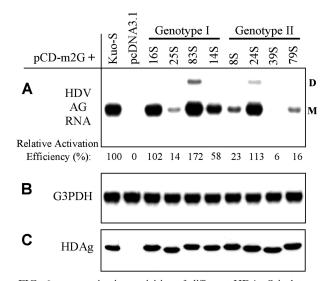


FIG. 6. trans-activating activities of different HDAg-S isolates on genotype I HDV replication. (A) Northern blot analyses of antigenomic (AG) RNA synthesis. HuH-7 cells in 6-cm-diameter dishes were cotransfected with a defective genotype I HDV dimeric DNA (i.e., pCD-m2G; 5 µg) and a plasmid coding for HDAg-S (5 µg) as indicated. Plasmid pcDNA3.1 was used as a control. Cellular RNA extracted on day 6 posttransfection was separated in formaldehyde gels, transferred to nylon membranes, and hybridized with  $[\alpha^{-32}P]CTP$ labeled genotype I HDV genomic-sense RNA. M and D, monomeric and dimeric forms of HDV antigenomic RNA, respectively. RNA levels were quantified by radioanalytic imaging. Values under lanes are the relative activation efficiencies of the clones compared to that of the prototype HDAg-S (Kuo-S). (B) Controls of RNA loading in each lane use glyceraldehyde-3-phosphate dehydrogenase as a reference. (C) Expression of HDAg-S in each sample. The cell lysates were analyzed by Western blotting using human anti-HDV.

inability to produce the antigenomic RNA transcripts 6 days after transfection (Fig. 6A, lane 2). However, antigenomic RNA was readily detected 6 days after cotransfection of pCDm2G with the cognate HDAg-S, i.e., Kuo-S, expression construct, pCMV-S (Fig. 6A, lane 1). In similar experiments, eight HDAg-S expression clones of either genotype I or II were individually cotransfected with pCD-m2G and examined for the trans-activation of HDV RNA replication. All HDAg-Ss were present intracellularly at comparable levels (Fig. 6C), but HDV RNA replication was trans-activated by the different HDAg-Ss to a wide variety of levels, ranging from 6 to 172% compared to the Kuo-S control (Fig. 6A). Among the four genotype I isolates, 16S had a trans-activation activity equivalent to that of Kuo-S. In contrast, the trans-activation activity of 83S increased to 1.7 times than that of the control, whereas those of 25S and 14S decreased to one-seventh and one-half that of the control, respectively. Interestingly, among the four isolates of genotype II, only 24S had a slightly higher transactivation activity than the control, and the remaining three were weaker and had a markedly reduced trans-activation activity. Nonetheless, all HDAg-S molecules supported HDV RNA replication to various degrees. Judging from the tested eight clones of HDAg-S, it seemed that HDAg-Ss of genotype I isolates were more likely to be strong supporters of genotype I HDV RNA replication than HDAgs-Ss of genotype II. Careful examination of sequence homology and a comparison with the relative trans-activation activities of HDV RNA replication

by different HDAg-Ss gave rise to no simple obvious correlation. Since these results were verified in three separate transfection experiments and since another HDAg-S cDNA clone (i.e., a quasispecies) derived from patient TW2476 produced similar observations, it is suggested that genetic distance alone is not a predictor of the degree of HDV RNA replication *trans*-activated by a given HDAg-S.

trans-activation of HDAgs-S on HDV replication of genotype II. The construct pCD-79mD2G was first created as a counterpart of pCD-m2G, from which genotype II HDV RNA replication could be activated by an HDAg-S-expressing plasmid. Without cotransfection with the expression plasmids for HDAg-S, pCD-m79D2G indeed yielded no evidence of HDV RNA replication in cells, as no antigenomic transcript was detected 6 days posttransfection. Upon cotransfection with an expression construct for genotype I or genotype II HDAg-S, the activation of HDV RNA replication was observed with some isolates at various efficiencies, ranging from 22 to 250% (Fig. 7) when the cognate HDAg-S (79S) was used as a reference. The antigenomic RNA was easily detected with three isolates of genotype II HDAg-S (8S, 24S, and 79S) and was barely detected with isolate 39S. In contrast, none of the four isolates of genotype I HDAg-S activated this genotype II HDV RNA replication to a significant level.

The region in HDAg-S that determines *trans*-activating efficiency. Within genotype I clones, 25S appeared to be the weakest supporter of RNA replication by transfection of pCD-m2G, whereas 16S was highly compatible and activated RNA replication indistinguishably from the cognate HDAg-S. To identify which regions of 25S and 16S are responsible for their different compatibilities (Fig. 6A), chimeras based on two arbitrary crossover points, residues 56 and 163, were constructed. For clarity of nomenclature, the chimeras are designated as follows.  $16N_{56}25CS$  represented an HDAg-S having residues 1 to 56 from 16S and the rest from 25S. Similarly,  $25N_{56}16CS$  contained residues 1 to 56 from 25S and residues 57 to 195 from 16S. Upon cotransfection with pCD-m2G,  $25N_{56}16CS$  and  $25N_{163}16CS$  both showed weak *trans*-activation activities similar to that of 25S (Fig. 8, compare lanes 2, 4,

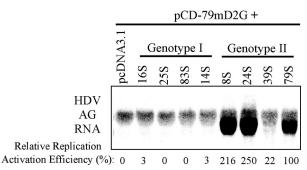


FIG. 7. trans-activating activities of different HDAg-S isolates on genotype II HDV replication. Cotransfection was carried out as described for Fig. 6 except that the HDAg-S-expressing plasmid (1  $\mu$ g) was paired with the construct carrying defective genotype II HDV dimeric DNA, pCD-79mD2G (5  $\mu$ g). Northern blot analyses of antigenomic RNA synthesis were performed also as for Fig. 6 except for probing with [ $\alpha$ -<sup>32</sup>P]CTP-labeled genotype II HDV genomic-sense RNA. Values under lanes are the relative activation efficiencies of the clones compared to that of the cognate HDAg-S (79S).

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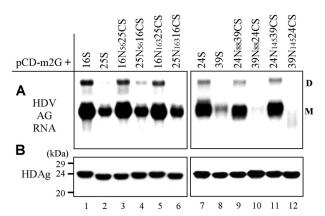


FIG. 8. trans-activation of RNA replication by chimeric HDAg-S constructs. Chimeric HDAg-S constructions are described in Materials and Methods. HuH-7 cells were cotransfected with pCD-m2G and the chimeric HDAg-S construct as indicated. Total RNA was isolated from these cells on day 6 posttransfection, and 15 μg of RNA was used in each lane. Northern blot analysis was done as for Fig. 6A. (B) Cell lysates in panel A were analyzed for the expression of HDAg-S chimeras by Western blotting using human anti-HDV.

and 6). On the other hand,  $16N_{56}25CS$  and  $16N_{163}25CS$ , like 16S, were strong trans-activators of HDV RNA replication. Thus, the swapping of a minimum of 56 residues in the Nterminal portion of HDAg-S is associated with the characteristic of being a strong or weak trans-activator of HDV RNA replication. To determine if HDAg-S of genotype II also has this property, 24S and 39S, with opposite properties (Fig. 6A), were used to create chimeras. Chimeras based on two arbitrarily crossover points, residues 88 and 145, were constructed. As shown in Fig. 8, 24N<sub>88</sub>39CS and 24N<sub>145</sub>39CS behaved like 24S (lanes 7, 9, and 11) whereas  $39N_{88}24CS$  and  $39N_{145}24CS$ were more like 39S (lanes 8, 10, and 12). Therefore, the Nterminal portion of HDAg-S determines the relative ability to trans-activate HDV replication. To rule out the possibility that the above observations could arise from different levels of HDAg-S expression, all the cellular lysates were analyzed for the presence of HDAg-S. Figure 8B shows that all the HDAg-S chimeras were expressed to comparable levels.

### DISCUSSION

HDAg-S is known to oligomerize either homotypically or heterotypically with HDAg-L in isolates of genotype I (5, 8, 22). It is believed, although not experimentally proved, that these oligomerizations occur also in HDAgs of genotype II. Since the viral nucleotide sequences vary by 30 to 40% among isolates of different genotypes (28, 37), it was not clear whether these diversified HDAg molecules of different genotypes, as in the cases of mixed infections (39), can associate with each other. For the first time here, we have provided evidence that HDAg interactions can readily occur in various combinations and occur without genotypic discrimination. One line of evidence presented above is the coprecipitation of two HDAg molecules simultaneously expressed in transfected HuH-7 cells. The second line of evidence is the presence of HDAg-S in VLPs formed by incorporation of HDAg-L into HBsAgformed particles. After close examination of the HDAg sequences, the results may be explicable by variations in HDAgs that do not alter the critical hydrophobic residues in the heptad repeats (Fig. 1). Since the N-terminal sequences of genotype III HDAgs have a similar pattern of variations (2, 3), we suggest that HDAgs of genotype III may undergo oligomerization in a similar manner.

In cultured cells, HDV RNA replication requires the expression of HDAg-S. Transfection of HDV cDNA constructs defective in HDAg expression provides the system by which the effect of homogenous HDAg-S molecules acting on the replication of HDV RNA can be observed (6, 18). In this system, all our HDAg-S clones (derived from patients) have shown some degrees of trans-activating activity on replication of genotype I HDV RNA. However, it was consistently concluded that the strength of trans-activation varied among isolates (3). On the other hand, the matching by genotype of HDAg-S and HDV RNA did not necessarily result in a strong activation of replication. Among the four clones of genotype I HDAg-S tested for their trans-activation activity on the replication of pCDm2G, two clones showed strong activity, one showed moderate activity, and the remaining one showed weak activity. In contrast, three out of four of the genotype II HDAg-Ss were weak trans-activators and only one was a strong trans-activator. When tested for the activation of replication of genotype II HDV RNA, i.e., cotransfected with pCD-79mD2G, none of the four isolates from genotype I HDAg-S clearly showed activity. In contrast, within the four isolates of genotype II HDAg-S, three clones (8S, 24S, and 79S itself) were strong trans-activators and only one (39S) had a low activity.

As shown from the above results on the support of RNA replication by HDAg-Ss with both genotypes I and II, 24S (a genotype II clone) was always a strong trans-activator whereas a second genotype II clone, 39S, was always a weak transactivator. These facts indicate there are substantial differences among clones of the same genotype. Although the clone number used in this study is still relatively small, it seems that there is no genotype restriction and that HDAg-S of genotype I is more likely to be a strong trans-activator of genotype I HDV RNA replication than HDAg-S of genotype II. This tendency, however, does not apply to the activation of genotype II HDV RNA replication. The replication activation of genotype II HDV RNA tends to have more genotype restriction than that of genotype I, and none of our genotype I HDAg-S clones was able to show the trans-activating ability to support genotype II HDV RNA replication.

These results differ slightly from the strict genotype-specific complementation that had been found for HDAg-Ss of genotypes I and III (3). Perhaps the greater divergence of HDV between genotypes I and III than between genotypes I and II accounts for this difference. It was recently proposed that the secondary structure of the HDV genome for HDV genotype I might be different from that for genotype III and that this could affect the RNA editing (1). The HDV RNA editing efficiencies for genotypes I and II also differed (13). A simple model then hypothesizes that HDV RNA of different genotypes may assume slightly different secondary structures, and the secondary structure of genotype I HDV RNA might be in a favorable configuration for HDAg-S activation compared to that of genotype II. To explain the observed differences among clones of the same genotype, the interactions among HDV

RNA, HDAg-S, and recruited replication factors must all be considered. Subtle differences in the molecular structures would influence their mutual interactions and consequently affect the efficiency of activation.

The question of which region of HDAg-S could predict a strong trans-activator of HDV RNA replication was addressed by fragment swapping between 16S and 25S as well as between 24S and 39S. All the results indicated that the N-terminal region of HDAg-S (with a minimum of 56 residues) determines the strength of trans-activation. This region covers the coiledcoil domain of HDAg-S that is required for trans-activation of HDV RNA replication and that is responsible for oligomerization (43, 45). By sequence comparison of the first 56 residues, it was found that there are nine amino acid differences between isolates 16S and 25S, and only one residue difference involves charge variation. There are also nine amino acid variations between 24S and 39S within this region, but no charged residues are involved. Intriguingly, no particular pattern could be deduced from these variations. We have examined whether these isolates have different RNA binding activities using a Northwestern blot assay, and no obvious differences were detected. One speculation that may explain our results is that an N-terminal region of HDAg-S with a strong trans-activation activity could facilitate the formation of a high-order structure activation complex and that this promotes HDV RNA replication. In contrast, those with weak trans-activation activity could be inefficient in this activity or could even hinder the cognate HDAg-S from so doing, and this could result in poor and inefficient activation HDV RNA replication.

An unexpected recombination might occur intracellularly due to our cotransfection of two plasmids carrying similar DNA sequences. If so, the viral RNA and HDAg might lose their genotype identities. Although this possibility could not be excluded completely, three lines of evidence suggest that it was unlikely to have happened in our experiments. First, in our coprecipitation assays, many pairs of plasmids were transfected to express HDAgs that were in turn analyzed by Western blotting using a genotype-specific antibody. The data (Fig. 2 to 5) indicated that the antigens produced were all discernible, and, in many cases, the antigen identities could be simply recognized by their characteristic sizes. Second, in our initial RNA replication analyses, both HDAg-S and viral RNA were produced intracellularly by simultaneous transfection of plasmids. However, the RNA products harvested from the transfected cells were reverse-transcribed into DNA, which was subsequently sequenced, and no recombination product was observed. Finally, to exclude possible DNA-DNA recombination, we recently performed transfection with an HDAg-Sexpressing plasmid coupled with in vitro-transcribed viral RNA that was derived from a second genotype but that was defective in HDAg-S production. Preliminary observations were similar to those based on two-plasmid DNA transfection (data not shown). These facts together suggest that our results are conferred by the genotype effect rather than caused by DNA recombination.

In conclusion, based on our limited strains, we found that HDV antigens are able to interact with each other without size and genotype differences and that HDAg-Ss of genotypes I and II are more clone dependent in the complementation for the *trans*-activation of genotype I HDV replication. On the other

hand, the *trans*-activation of genotype II HDV RNA replication seems to strictly require HDAg-S of genotype II. However, more clones of HDV and HDAg-S need to be examined to consolidate this notion.

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